Z. klin. Chem. u. klin. Biochem.
10. Jg. 1972, S. 215—219

A New Enzymatic Method for the Estimation of Inorganic Phosphate in Native Sera

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(Eingegangen am 6. September 1971)

A new enzymatic method for the determination of inorganic phosphate (P_I) is described. It utilizes a reaction of the Embden-Meyerhof pathway in which triosephosphate takes up P_I to form glycerate-1,3-diphosphate. It was developed for use with nondeproteinized sera containing high amylase activity. The previously described method based on phosphorolysis of glycogen is not satisfactory in such instances and is only half as sensitive as the new method. As the increased sensitivity requires purer reagents, a procedure for removal of contaminating inorganic phosphate and pyrophosphate is given.

Es wird eine neue enzymatische Methode für die Bestimmung von anorganischem Phosphat (P_I) beschrieben. Sie beruht auf der Reaktion des Embden-Meyerhof-Weges, in der Triosephosphat anorganisches Phosphat aufnimmt und Glycerat-1,3-diphosphat bildet. Sie wu de für nicht enteiweißte Seren mit hoher Amylaseaktivität entwickelt. Die zuvor beschriebene Methode, die auf der Phosphorolyse von Glycogen beruht, ist hierfür nicht brauchbar und nur halb so empfindlich wie die neue Methode. Da die höhere Empfindlichkeit reinere Reagenzien erfordert, wird eine Vorschrift für die Entfernung von anorganischem Phosphat und Pyrophosphat als Verunreinigung angegeben.

An enzymatic method for the estimation of inorganic phosphate (P_i), previously reported from this laboratory (1), utilized glycogen and the enzyme phosphorylase[1]) to esterify P_i. The product of this reaction, glucose-1-phosphate, converted via glucose-6-phosphate to gluconolactone-6-phosphate, was measured by the amount of NADP reduced. This method, which will be referred to hereafter as the glycogen method, is very satisfactory for the analysis of protein-free extracts of tissues as well as native, i. e. not deproteinised human sera. It is unsatisfactory, however, for use with native sera of such common experimental animals as dogs, rats, etc., as these have a very high amylase activity. Amylase breaks down glycogen and thus "consumes" part or most of the substrate that ought to be present in excess throughout the determination. To circumvent the above difficulty a new method was developed which utilizes the second reaction of the glycolytic pathway where P_i is esterified at substrate level, namely, the reaction of triosephosphate dehydrogenase:

(1) P_I + triosephosphate + NAD^+ $\xrightarrow{\text{triosephosphate dehydrogenase}}$ glycerate-1,3-diphosphate + NADH + H^+

Coupled with it are the following reactions:

(2) glycerate-1,3-diphosphate + ADP $\xrightarrow{\text{phosphoglycerate kinase}}$ glycerate-3-phosphate + ATP

(3) ATP + glucose $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP

(4) glucose-6-phosphate + $NADP^+$ $\xrightarrow{\text{glucose-6-phosphate dehydrogenase}}$ gluconolactone-6-phosphate + NADPH + H^+.

In the first reaction the esterification of P_i results in reduction of NAD. The product of esterification donates its phosphate to ADP in reaction (2). Reaction (3) and (4) make use of the ATP generated to reduce NADP. Therefore, for every mole of P_i esterified, 1 mole of NAD and another of NADP are reduced. Originally, glyceraldehyde-3-phosphate served as substrate. Later on, glyceraldehyde-3-phosphate was generated from dihydroxyacetone phosphate, and finally from fructose-1,6-diphosphate, which proved even better. Each of the three glycolytic intermediates gives satisfactory results, but we have abandoned the use of the trioses since fructose-1,6-diphosphate is stable and more economical.

Finally, it is important to emphasize that the new method, which will be referred to as the triosephosphate method, measures not only P_i, but also inorganic pyrophosphate (PP_i). This is due to the fact that commercially available hexokinase (see reaction 3) is contaminated with inorganic pyrophosphatase. In our experience native sera do not contain any PP_i nor its phosphatase. In tissues, however, PP_i undoubtedly plays an important role. In this communication, therefore, we discuss the triosephosphate method only as applied to sera, and data on P_i and PP_i content of tissues will be published separately.

[1]) Enzymes: Phosphorylase (EC 2.4.1.1), Amylase (EC 3.2.1.1), Triosephosphate dehydrogenase (EC 1.2.1.12), Phosphoglycerate kinase (EC 2.7.2.3), Hexokinase (EC 2.7.1.1), Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), Inorganic pyrophosphatase (EC 3.6.1.1), Triosephosphate isomerase (EC 5.3.1.1), Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), Aldolase (EC 4.1.2.13), Alkaline phosphatase (EC 3.1.3.1), Lactate dehydrogenase (EC 1.1.1.27), Fructose-1,6-diphosphatase (EC 3.1.3.11), Phosphoglucomutase (EC 2.7.5.1).

Experimental

Preparation of samples for analysis

Samples of blood from experimental animals were obtained by heart puncture and transferred to narrow plastic centrifuge tubes, capacity ca. 0.5 ml. Fifteen min. at room temperature were allowed for clotting. Several of the plastic tubes were tightly packed in a regular size centrifuge tube and centrifuged for 15 min. at 2000 rpm. Human samples were collected from a drop of blood by means of a Marburg Microliter pipette of 50 μl capacity (obtainable from Eppendorf Gerätebau, Hamburg, Germany). The plastic tip was plugged with plasticine to prevent leakage, then disconnected from the pipette. After clotting of the blood, the tip was placed in a round-bottom centrifuge tube, cushioned with plasticine and centrifuged.

Reagents and their purification

All reagents were obtained from Boehringer - Mannheim (Germany) except for EDTA and glycogen which were purchased from Merck Co. (Darmstadt, Germany). Glass redistilled water was used for preparing solutions and for rinsing glassware.

The success of the triosephosphate method depends mainly upon the quality of the reagents, hence the necessity of choosing them carefully. The degree of contamination with phosphates must be checked, and this is best done by the method of FISKE and SUBBAROW (2). In our experience, the following products of Boehringer contain more phosphate than is permissible: glucose-6-phosphate dehydrogenase, phosphoglycerate kinase and fructose-1,6-diphosphate. From these, the contaminating phosphate is precipitated with magnesium acetate. NADP and ADP contain varying amounts of P_i, but it is possible to select suitable preparations. ADP powder may also contain PP_i, thus it is recommended that ADP solutions be tested by the glycogen method (1, 3). The latter measures P_i and, after addition of inorganic pyrophosphatase, also PP_i. If one of the trioses, namely glyceraldehyde-3-phosphate or dihydroxy acetone phosphate, is to be used as substrate instead of fructose-1,6-diphosphate, its commercial derivative must be carefully hydrolysed. Prolonged hydrolysis as well as prolonged storage increase P_i due to partial decomposition of the free, labile ester.

Hydrolysis of commercial triosephosphates

Glyceraldehyde-3-phosphate: Suspend 50 mg of D,L-glyceraldehyde-3-phosphate diethylacetal (Ba salt) in 2 ml of H_2O in a glass-stoppered centrifuge tube. Add 1 g of dowex-50 H^+ (100 to 200 mesh), shake vigorously until all powder goes into solution, centrifuge and decant supernatant. Wash dowex residue with 0.6 ml H_2O and add washings to the main portion. The combined solutions, which have a pH of around 1.5, are incubated at 39° to 40° for 18 hours only. Determine D-glyceraldehyde-3-phosphate (with triosephosphate isomerase and glycerol-3-phosphate dehydrogenase) and analyze for P_i (the contamination with the latter should not exceed 50 μg/ml). Store the acidic solution of glyceraldehyde-3-phosphate frozen and use within 2 months. The triosephosphate method calls for ca. 0.2 μmole of D-glyceraldehyde-3-phosphate per P_i determination carried out in a volume of 1.2 ml.

Dihydroxyacetone phosphate: Dissolve 15 mg dihydroxyacetone phosphate dimethylketal, dicyclohexylammonium salt · H_2O in 2 ml of H_2O, add 0.3 g dowex-50 H^+, shake for a few minutes, centrifuge and decant. Wash dowex residue with 0.6 ml H_2O, combine washings with the main portion and let stand at 39°—40° for 4 hours only. Solutions so prepared have a very low initial P_i content. They must be analysed (glycerol-3-phosphate dehydrogenase) and used within a month of frozen storage. The amount required is 0.45 μmole per determination (vol. 1.2 ml).

Procedure for precipitation of contaminating phosphates

Magnesium ammonium phosphates are practically insoluble. This property serves as a basis for purifying the reagents. To enzymes, which are as a rule suspended in $(NH_4)_2SO_4$, one simply needs to add an excess of Mg^{++}. Magnesium acetate is most suitable for this purpose, as its anion is harmless, and it also provides a convenient pH of around 7.5 at which the precipitations are best carried out. This technique of precipitation is superior to dialysis or washing procedures usually applied to enzymes. In our experience it is also superior to recrystallizations of a substrate such as fructose-1,6-diphosphate.

Glucose-6-phosphate dehydrogenase. The Boehringer suspension of 5 mg enzyme protein/ml usually contains ca. 1 mg P_i/ml and most of it is removed as follows: Mix in a small centrifuge tube 100 μl of the enzyme and 400 μl of 0.4M magnesium acetate. Place the tube in ice and scratch the wall with a glass rod for 5—7 min. to initiate precipitation. Keep for additional 15 min. in ice and centrifuge in the cold. Analyze supernatant for P_i content and assay enzyme activity.

Recently, we have obtained from Boehringer a preparation of glucose-6-phosphate dehydrogenase which contains as little as 1/5th the usual amount of P_i. This product is suitable for direct use with the glycogen method but still unsuitable for the triosephosphate method, as the latter is more sensitive. Since it is more difficult to get rid of contaminating P_i when its initial concentration is so low, follow the procedure described above, but allow 2 to 3 days for precipitation with occasional scratching. If it is not convenient to wait so long, substitute 0.8M magnesium acetate for the usual 0.4M and the precipitation will not require more time than 15 min. as in the case of the more highly contaminated enzyme. In our experience the magnesium acetate treated glucose-6-phosphate dehydrogenase is as stable as the original suspension.

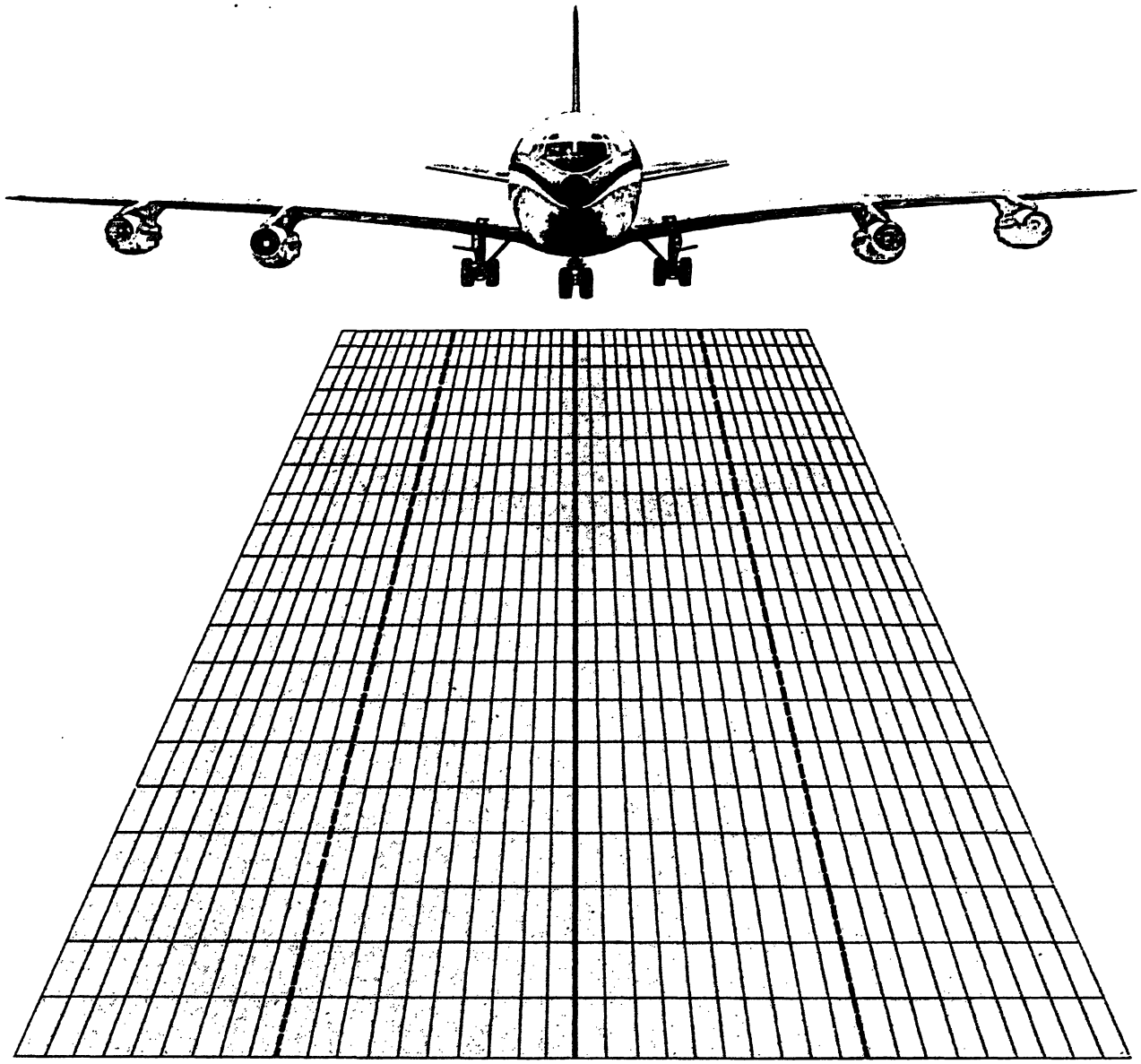
Phosphoglycerate kinase. The Boehringer product is a suspension in $(NH_4)_2SO_4$ and 0.04M $Na_4P_2O_7$. According to our analyses over the past few years, 1 ml containing 10 mg enzyme protein, gives between 2.0 and 2.5 mg P_i upon hydrolysis in 1N HCl, around one tenth of it being true P_i. It is necessary to free the enzyme from PP_i, as hexokinase, which is used in the P_i assay system, has inorganic pyrophosphatase activity. The following procedure removes both the PP_i and the small amount of P_i: Mix in a centrifuge tube 200 μl phosphoglycerate kinase, 10 mg enzyme protein/ml, and 300 μl of 0.4M magnesium acetate. Immerse in ice for 5—7 min., scratch the wall of the tube and let stay in refrigerator for 1—3 days. Stir the precipitate, scratch again, let stand for 15 min. in ice to complete the precipitation and centrifuge in the cold. Analyse the supernatant for P_i after hydrolysis in 1N HCl (method of FISKE and SUBBAROW). If the hydrolyzable phosphorus is low enough, separate the supernatant. Otherwise, stir and keep an additional day or so in a refrigerator, centrifuge and analyze. The concentration in the supernatant is 4 mg enzyme protein/ml.

The newest catalogue of Boehringer states that their phosphoglycerate kinase is suspended in $(NH_4)_2SO_4$ and 1 mM EDTA, but no mention is made of PP_i, as was the case in the past. We have obtained most recently such a preparation and found that 1 ml of 10 mg enzyme protein contains slightly over 0.5 mg of P_i and 0.2 mg of PP_i phosphorus. The procedure which is recommended for the purification of such a product, is to mix 100 μl of the enzyme suspension with 400 μl of 0.8M magnesium acetate and allow 1—3 days for precipitation. Using these proportions one gets a supernatant of 2 mg enzyme protein/ml.

As a rule, phosphoglycerate kinase must be tested for activity to make sure that a good excess is provided to drive the two preceding reactions, namely, that of aldolase and triosephosphate dehydrogenase, in the forward direction.

Fructose-1,6-diphosphate. Dissolve 50 mg fructose-1,6-diphosphate tetra-hexylammonium salt · 10 H_2O in 250 μl of 0.8M magnesium acetate, let stand for 10 min., add 250 μl of 1M $(NH_4)_2SO_4$, put in ice and scratch the wall of the centrifuge tube for 5—7 min. with a glass rod. Put in refrigerator and keep for 1—2 days with occasional scratching (3—4 times). Allow 15 min. in ice after last scratching, centrifuge in the cold. Analyze supernatant for P_i and determine fructose-1,6-diphosphate enzymatically using aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase.

Auf sicherem Kurs



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A P_i determination carried out in a volume of 1.2 ml requires a third of a μmole of fructose-1,6-diphosphate as substrate. This amount is generally found in 3 μl of the supernatant. The total contamination in these 3 μl after the above treatment is around 0.1 μg of P_i. The commercial fructose-1,6-diphosphate may contain 10—20 times as much and is unsuitable for direct use.

Enzymatic Method

All measurements are done using a Beckman spectrophotometer model B at 340 nm. Semi-micro cuvettes, light path 10 mm, width 4 mm, are used. All P_i determinations are carried out in a final volume of 1.2 ml. Marburg microliter pipettes are used for delivery of small volumes of reagents, enzymes and samples.

Five commercially available enzymes are required and it is advisable to use the concentrated preparations in order to minimize the amount of $(NH_4)_2SO_4$ in the assay system. Two highly contaminated enzymes, glucose-6-phosphate dehydrogenase and phosphoglycerate kinase, need to be purified (see preceding directions). Preparations of hexokinase being slightly contaminated, should be analyzed for P_i and the best product selected. The remaining two enzymes, aldolase and triosephosphate dehydrogenase, contain as a rule no impurities of phosphates. Procedures for measuring enzyme activities are provided by the Boehringer firm.

The triosephosphate method operates more efficiently at an alkaline pH. The starting pH of the buffer required is 8. As many reagents are added, like NAD, NADP and the 5 enzymes, the pH decreases and reaches around 7.7. Acidic reagents must be avoided, because if the pH during the actual measurement is less than 7.7, the system does not operate quantitatively. With each set of determinations it is necessary to carry a reagent blank and a standard through out the whole procedure. The reagent blank, which contains all the reagents and enzymes, measures their contamination with phosphates. The standard cuvette indicates with what efficiency the system operates and whether the esterification of P_i is quantitative.

Procedure: The P_i determinations are carried out in 1.2 ml of 50 mM triethanolamine containing 5 mM EDTA. The method calls for the following concentrations of reagents in the above buffer: 1 mM glucose, 0.02 mM ADP, 0.5 mM NAD, 0.33 mM NADP and 5 mM magnesium acetate. The native sera are added in an amount of 10 μl or less and kept for 15 min. in order to allow for inactivation of the serum enzymes. This is followed by introducing the enzymes and finally the assay is started by adding the substrate, namely fructose-1,6-diphosphate. If glyceraldehyde-3-phosphate or dihydroxyacetone phosphate is to replace the latter, see "alternative procedures".

For convenience it is suggested that the following reagents be mixed: 500 μl Triethanolamine-EDTA buffer pH 8 (Triethanolamine 120 mM, EDTA 12 mM), 40 μl glucose (30 mM), 10 μl ADP (2.4 mM, ADP Na_3), 30 μl magnesium acetate (200 mM), 10 μl NAD (60 mM), 10 μl NADP (40 mM, NADP Na). These quantities add up to 600 μl and are needed per one determination. Prepare enough reagent mixture to pipette 600 μl for reagent blank, a standard and each of the unknowns. The volume of enzymes, native serum and substrate required must add up with H_2O to another 600 μl. Pipette the reagent mixture, the calculated amount of H_2O, the standard and the sera into appropriate cuvettes. Allow 15 min. for inactivation of enzymes in sera and during that time prepare enzyme mixture. The following quantities of each enzyme are required per determination: 5 μl glucose-6-phosphate dehydrogenase, 1 mg enzyme protein/ml or amount equivalent to 0.35 units; 5 μl hexokinase, 2 mg enzyme protein/ml if activity not determined, otherwise 0.8 units; 3 μl phosphoglycerate kinase, 4 mg enzyme protein/ml, or amount equivalent to 5 units; 5—7 μl triosephosphate dehydrogenase, 10 mg enzyme protein/ml if activity has not been determined, otherwise an amount equivalent to 2.5 units; 5—6 μl aldolase, 10 mg enzyme protein/ml, if activity has not been determined, otherwise 0.35 units. Prepare enough mixture for all cuvettes and if desired, include some H_2O to get a convenient volume for fast delivery (Marburg Pipette). Introduce the enzyme mixture into all cuvettes, measure the absorbance (E) at 340 nm (wait until steady readings are obtained), then start the assay by adding fructose-1,6-diphosphate (0.33 μmole in a volume of 20 μl). Take measurements every 5 min. for the next 20—30 min. A standard of 0.03 μmole P_i usually gives a ΔE of 0.310 within 10—15 min. This value is obtained after correction for contaminating phosphates in reagents and enzymes, and it remains steady although the reactions do not come to a complete stop due to some phosphatase impurity present in the enzyme mixture. The ΔE values for sera, as calculated by subtracting the corresponding ΔE for the reagent blank, reach also the maximum within 15 to 20 min. and remain steady.

For calculations, let us consider a determination done on rat serum. Twenty min. after adding fructose-1,6-diphosphate, the reagent blank gave a ΔE of 0.155, the standard a ΔE of 0.465 and the rat serum a ΔE of 0.385. By subtracting 0.155 from each of the two values, we obtain $\Delta E = 0.310$ for standard, which is theoretical and $\Delta E = 0.230$ for rat serum. Since 10 μl of the serum were used for this determination, the content of P_i in mg/l of rat serum is:

$$\frac{0.230}{6.22} \cdot 1.2 \cdot \frac{31}{2} \cdot 1000 \cdot \frac{100}{1000} = 68.8$$

(6.22 being the μmolar extinction coefficient for NADH and NADPH at 340 nm).

General Remarks and Precautions

According to the directions given, the magnesium acetate concentration in the buffer is 5 mM. This does not include the extra magnesium introduced with the two enzymes and fructose-1,6-diphosphate which are purified by the precipitation procedure. If the enzymes are treated with 0.8 instead of 0.4M magnesium acetate to remove phosphates, the Mg^{++} used in the buffer must be reduced to one half. Excess of this ion makes EDTA ineffective and thus if a serum exhibits high alkaline phosphatase activity, some P_i splits slowly and gives a gradual increase for P_i value of that serum (EDTA inhibits alkaline phosphatase).

Another difficulty is that sera with a very high pyruvate content may show a back-run. A small amount of lactate dehydrogenase present in the system causes a slow reduction of pyruvate and thus a reoxidation of NADH. This was observed with blood serum obtained from a dog heart-lung preparation after severe anoxia. In such a case one may add some NADH and lactate dehydrogenase before fructose-1,6-diphosphate so as to convert all the pyruvate in the sample to lactate before the actual determination is started.

The use of hemolyzed sera should be avoided as these show high triosephosphate isomerase activity. This enzyme catalyses dihydroxyacetone phosphate formation from glyceraldehyde-3-phosphate, the equilibrium being in favor of the former. Thus, part of the glyceraldehyde-3-phosphate generated from fructose-1,6-diphosphate becomes unavailable for esterification of P_i and the system may not operate quantitatively. If only hemolyzed sera are available, we recommend use of dihydroxyacetone phosphate as starting substrate according to directions given under "Alternative Procedures".

The procedure utilizing dihydroxyacetone phosphate or glyceraldehyde-3-phosphate as substrate should also be applied to analyses of sera which exhibit fructose-1,6-diphosphatase activity or in case one of the commercial enzymes used for P_i determination happens to be contaminated with this phosphatase.

Aldolase cleaves fructose-1,6-diphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The latter remains unutilized during the determination and it is very important that the commercial enzymes are not contaminated with glycerol-3-phosphate dehydrogenase. One way to avoid the interference of this enzyme would be to carry out the P_i determination starting with glyceraldehyde-3-phosphate as substrate.

Alternative Procedures

In some special cases it may be necessary to use one of the trioses as substrate rather than generate it from fructose-1,6-diphosphate.

The main inconvenience lies in the conversion of the commercial triose into one suitable for use. In case of glyceraldehyde-3-phosphate, the starting product is an insoluble Ba salt. After Dowex treatment, 18 hours are required to split off the protective ethyl groups. The commercial product of dihydroxyacetone phosphate is a cyclohexylammonium salt. It is soluble and takes only 4 hours to split off the protective groups, but it is far too expensive for routine work. Both free esters are rather unstable and must be frequently analyzed.

The procedure for P_i determinations using glyceraldehyde-3-phosphate or dihydroxyacetone phosphate as substrate remains the same as when fructose-1,6-diphosphate is used, except for the following minor variations:

If one starts with glyceraldehyde-3-phosphate, the magnesium acetate concentration in the buffer is increased from 5 to 6.3 mM and aldolase is omitted from the enzyme mixture.

If one starts with dihydroxyacetone phosphate, the initial pH of the buffer is raised to pH 8.5, magnesium acetate concentration in this buffer is increased to 6.3 mM and 5 µl of triosephosphate isomerase, 2 mg enzyme protein/ml, are added together with the other enzymes. Aldolase is excluded from the enzyme mixture.

Results and Discussion

The colorimetric method of FISKE and SUBBAROW requires that sera be deproteinized and therefore relatively large samples of blood are needed. This is wasteful when human blood is used and renders experimentation in the case of small animals difficult or impossible. The enzymatic methods are not only more sensitive, but they can be applied to nondeproteinised sera. The glycogen method described earlier, gives excellent results for humans, with a few exceptions. One needs as little as 20 µl of serum for a determination. The need for an enzymatic micro-method utilizing a substrate other than glycogen, which would be applicable to laboratory animals, is obvious. The triosephosphate method is the answer to this problem. One reason why it has not been worked out so far, may lie in the unavailability of the proper reagents and enzymes, for although they are all commercially available, their contamination presents serious problems. The inconvenience of preparing free trioses from the commercial products and their relative instability, can be overcome by generating them from fructose-1,6-diphosphate. Theoretical considerations do not at first sight make this appear possible, as the aldolase reaction which cleaves fructose-1,6-diphosphate has a very unfavorable equilibrium for the formation of the trioses; also, reaction (1), which is supposed to esterify P_i quantitatively, does not proceed readily to the right. In nature, however, glycolysing tissues face the same unfavorable equilibria under suboptimal conditions, yet readily produce lactic acid. The new method was made to succeed by the application of alkaline pH which favors the forward direction of reaction (1). The products of this reaction were withdrawn by means of reactions (2), (3) and (4), the equilibria of which are all far to the right. Under such conditions the system operates quantitavely with fructose-1,6-diphosphate as substrate.

The triosephosphate method has been in use for over 2 years in our laboratory and checked routinely with the method of FISKE and SUBBAROW where volume of blood was not restrictive (dog). The maximum discrepancy between these two methods, amounts to no more than $\pm$ 6%. This accuracy applies also to the analysis of samples of orthophosphate in amounts differing up to one order of magnitude. Such an error is within the precision limits of the measuring instruments used. Numerous P_i determinations on human sera were performed utilizing the old and the new enzymatic method and checked against results obtained in the hospital laboratories by the method of FISKE and SUBBAROW. Again, agreement was satisfactory, except for sera that exhibit very high alkaline phosphatase activity. Under such circumstances, both enzymatic methods show a high esterification rate and a continuous increase in ΔE for P_i was observed. Upon addition of an inhibitor of alkaline phosphatase, such as phenanthroline or theophylline, the situation is corrected. The determinations of P_i on such sera have to be repeated, the inhibitor being included from the start in the cuvette mixture, to give a final concentration of 1 mM. The P_i values obtained by applying

Tab. 1
Analysis of native sera for P_i

Species	Amylase (SOMOGYI Units)	P_i, g/l		
		Methods: Glycogen*)	Triosephosphate	FISKE and SUBBAROW
Human, normal	150	31	31	30
Human, abnormal	3000	—	24	25
Dog	1200	—	40	40
Cat	1490	—	45	46
Rabbit	6	45	43	44
Rat	2650	—	93	91
Mouse	1550	—	75	76
Rat, Fluoroacetate treated (3 mg/kg, i. p.)	before injection	—	69	—
	1 h later	—	42	—
	2 h later	—	218	220

*) For the glycogen method the reagents are mixed in the following proportions: 500 µl triethanolamine-EDTA buffer pH 7.6 (triethanolamine 120 mM, EDTA 12 mM), 50 µl glycogen (2%), 10 µl NADP (40 mM), 5 µl AMP (50 mM), 5 µl glucose-1,6-diphosphate (1.2 mM) and 30 µl magnesium acetate (200 mM). Each determination requires 600 µl of this mixture, which with H_2O, 20 µl of serum and the necessary enzymes, add up to a final volume of 1.2 ml. Enzymes required are: 5 µl glucose-6-phosphate dehydrogenase (1 mg enzyme protein/ml or 0.35 U), 5 µl of phosphoglucomutase (2 mg enzyme protein/ml) and 25—35 µl of phosphorylase a (2 mg enzyme protein/ml, which is prepared as follows: weigh an amount equivalent to 2 mg enzyme protein of lyophylised phosphorylase a of Boehringer and dissolve it in 1 ml of 0.5% glycogen 24 h before use). A reagent blank and a standard must be carried along with each set of determinations as described for triosephosphate method.

this procedure, agree with those obtained by the use of deproteinised sera (method of FISKE and SUBBAROW). In experienced hands such observations are of diagnostic value.

The accompanying table illustrates some of our data. The first 2 examples are those obtained for human sera, normal and abnormal, regarding their respective amylase activity. Normal values for this enzyme in humans may reach up to 200 SOMOGYI units. The abnormal serum has an unusually high amylase activity and was withdrawn from a patient suffering from severe pancreatitis. This serum, when analyzed by the glycogen method, appeared to contain practically no P_i. Before the determination was terminated, extra glycogen was introduced into the cuvette and as a result, P_i present in the serum was esterified. Therefore, although the glycogen method is not applicable in cases of pancreatitis, it may be of diagnostic value.

The amylase values for sera of experimental animals quoted in the table, except for the rabbit, are all high and therefore the glycogen method can not be used. Results obtained by the triosephosphate method compare favorably with those of the method of FISKE and SUBBAROW.

Finally, the advantages of the method described may be exemplified by its application to a study on rats. Fluoroacetate, a known rodent poison, causes accumulation of citrate in various organs. As citrate binds Ca^{++}, one might expect a change in the P_i level of blood. In the experiment quoted, blood was analyzed for P_i before injection of fluoroacetate and at two subsequent intervals, as symptoms of poisoning developed and progressed. 0.1 ml of blood removed by heart puncture yields an amount of serum sufficient for several micro-determinations. (We often draw as many as five samples and collect a sixth, larger one, when the animal is sacrificed.) In the fluoroacetate experiment, the blood P_i at first decreases and later increases sharply. Not all rats respond so dramatically to this poison, but this case was chosen to illustrate the reliability of the method in case of wide variations in the P_i content of sera.

We gratefully acknowledge the competent assistance of Mrs. MELINE SEPETJIAN who helped with the precipitation procedures for the removal of contaminating phosphates.
This work was supported by a grant from the Lebanese National Research Council.

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